Detection of plot-level changes in ectomycorrhizal communities across years in an old-growth mixed-conifer forest

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Summary

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• Understanding spatial and temporal patterns present in ectomycorrhizal fungal community structure is critical to understanding both the scale and duration of the potential impact these fungi have on the plant community. While recent studies consider the spatial structure of ectomycorrhizal communities, few studies consider how this changes over time.
• Ectomycorrhizal root biomass and the similarity of community composition were measured at scales up to 20 cm replicated in nine plots and over 3 yr. Soil cores were additionally stratified into three depths.
• Annual occurrence of the dominant ectomycorrhizal species was constant at larger spatial scales but varied more across years at a fine spatial scale. Turnover of ectomycorrhizal species between years was observed frequently at scales < 20 cm. The ectomycorrhizal community within a plot was more similar across years than it was to other plots sampled in the same year.
• Our results demonstrate the dynamic nature of the ectomycorrhizal community even in the absence of large-scale disturbances. The potential role of root turnover and drought stress is discussed.

Introduction

Ectomycorrhizal (ECM) fungi can affect plant performance (Smith & Read, 1997) and play an important role in plant community structure through amelioration of stressful environmental conditions (Parke *et al.*, 1983; Horton *et al.*, 1999), mediation of competition (Perry *et al.*, 1989), and facilitation of regeneration following disturbance (Perry *et al.*, 1992). Despite a growing knowledge about ECM effects on plant communities, our understanding of the spatial and temporal dynamics of ECM communities is still limited (Read, 2002). Understanding these dynamics is critical to assessing both the scale and the duration of any taxon-specific ECM effects. Much of our knowledge about ECM community dynamics is based on the observations of fungal

sporocarps (Mason *et al.*, 1987; Nantel & Neumann, 1992; Matsuda & Hijii, 1998; Jumpponen *et al.*, 1999). However, sporocarp composition does not usually reflect below-ground composition (Gardes & Bruns, 1996; Jonsson *et al.*, 2000; Peter *et al.*, 2001) and therefore it remains unclear if this variation reflects below-ground fluctuations.

A number of studies have addressed aspects of temporal and spatial structure of ECM, but each of them have had important limitations, most likely due to the challenging nature of sampling these fungi (Horton & Bruns, 2001; Taylor, 2002). Temporally, ECM root abundance can be variable even across seasons (Harvey *et al.*, 1978; Swaty *et al.*, 1998) but because species identifications were not included in these studies, we do not know how individual fungal species are affected. The lifespan of ECM roots can span across many

years, and potentially varies across species (Treseder *et al.*, 2004), but it is not clear how that in turn affects overall community structure. Spatially, many studies have considered plot-level ECM community structure and have demonstrated that there is considerable species turnover across just 1–4 m or between trees (Gehring *et al.*, 1998; Jonsson *et al.*, 2000; Peter *et al.*, 2001; Tedersoo *et al.*, 2003; Lilleskov *et al.*, 2004). However, these studies were limited to single time points making it harder to generalize about the observed patterns. Additionally, many of these studies were restricted to the uppermost 5–10 cm of soil or did not stratify by depth. Therefore, they may have missed either a great deal of the overall diversity that can be found in deeper soil (Rosling *et al.*, 2003) or patterns driven by species that are partitioned by depth (Dickie *et al.*, 2002).

The old-growth mixed-conifer forest in the Sierra Nevada, California, presents a unique opportunity to understand natural levels of ECM species turnover. Fire suppression policies have kept an otherwise historically frequent large-scale disturbance effectively out of these forests for over 90 yr in many areas. ECM communities in these forests therefore are as close to a 'steady-state' as can be found. At the same time, disturbance may still come in other forms and scales. At the large scale, these forests experience predictable water stress in the form of an annual summer drought period. Such water stress is known to affect bacteria in surface soils (Fierer *et al.*, 2003) and therefore may affect ECM as well. At the small scale, the natural emergence of a fine root into soil space may also act as an enrichment disturbance and as a possible driver of diversity (Bruns, 1995).

The purpose of our study was to test for annual changes in ECM root activity and community composition in one such old-growth forest. Sampling was performed at distances that are as small as would be practical for field studies (5–20 cm), and spanned across multiple years. If drought acts as a direct disturbance to the fungi in this system it is expected to do so primarily in the soil closer to the surface therefore we stratified our sampling to be able to account for this. By including depth information and making extensive efforts to identify the ECM fungi we attempted to overcome some of the limitations presented in other studies. Additionally, by including and interconnecting this many variables, we expected a unique view into the dynamics of these fungi on the roots.

Methods

Site description

This study was conducted at Teakettle Experimental Forest (36°58′N; 119°2′W) – an old-growth mixed-conifer forest in the Sierra National Forest, California, USA. This site has been described in detail by North *et al.* (2002; also see http://teakettle.ucdavis.edu). Briefly, this is a montane forest (2100 m) on the south-western slope of the Sierra Nevada.

The overstory is composed primarily of four ectomycorrhizal species. *Abies concolor* and *A. magnifica* are dominant across the site based on relative basal area while *Pinus jeffreyi*, and *P. lambertiana* co-occur at lower frequencies. *Calocedrus decurrens*, an arbuscular mycorrhizal species, also occurs at high frequencies throughout the forest. Forest soils are classified as coarse and loamy (Xerumbrepts and Xeropsamments types), and have pH ranging from 5.2 to 6.0. Mean annual precipitation is 125 cm, most of which comes in the form of snow between November and May The average monthly rainfall between May and November for this region is < 2 cm. Air temperatures average 15.5°C in the summer and 0.7°C in the winter.

Sampling technique and justification

Soil sampling was set up to test ECM community structure and activity across space and time at small (0–20 cm) and large (25–200 m) spatial scales. Nine plots were established in the UN3 compartment between July 30–August 4, 1999. This compartment was chosen as a plot representative of typical forest structure and is one of many that were established for a multidisciplinary study on forest management (North *et al.*, 2002). Plots were chosen with the criteria of first being likely to have only *Abies* spp. roots in the sample, and second being as evenly distributed across the compartment as possible (see Fig. S1, available online as supplementary material). We attempted to limit the sampling to *Abies* spp. roots to reduce any potential variation in the fungal community that could be brought about by ECM host preference. To collect ECM roots, an initial soil core (2.8 cm^2) was taken 2 m from a large *Abies* spp. tree in a random direction. Another random direction was chosen and three additional cores were then taken 5, 10, and 20 cm in a line from the original core. Each soil core was separated into three depths: the O horizon, the first 10 cm of the mineral layer, and the second 10 cm of the mineral layer. Soils in the forest were noticeably drier in the upper layers of soil. For the next 3 yr (July 18–20, 2000; May 19–21, 2001; June 4–6, 2002) this sampling was repeated in a parallel line 5 cm away from the previous years cores. DNA from a number of samples from the 2000 season degraded and therefore this year was excluded from the final analyses. This left a total of 144 cores that were included in this study. The seasonal conditions were consistent across the 2001 and 2002 samplings that occurred immediately following the snowmelt. However, the 1999 sampling occurred later in the summer – 8–10 wk past the final snowmelt and at the height of the summer drought period typical in this region.

Sorting and handling of mycorrhizal tips

Soil samples were placed on ice immediately after collecting, transported back to the lab within 2 d and stored at 4°C.

Within 10 d of collecting, ECM roots were removed from the soil samples for processing. Total roots were initially washed and removed by rinsing the soils over a 0.35-mm sieve. Roots were determined to be living or dead based on the health of the stele. Roots were determined to be ectomycorrhizal based on the presence of a healthy fungal sheath, or if they exhibited morphological signs, such as swelling, coupled with visual presence of hyphae when viewed under a dissecting microscope. Almost all living roots were ectomycorrhizal. Within each core depth fraction, all actively ECM root tips were then divided into morphological types based on color, shape, and any distinctions in mantle morphology. Care was taken to split morphotypes when slight variations were found. No attempt was made to equate morphotypes between the core depth fractions to minimize the likelihood of mistakenly grouping types. Following morphotyping, roots were immediately frozen in water and freeze-dried. Dry root biomass was measured for each morphotype as our estimate of abundance.

DNA analysis of ECM fungi for taxonomic identification

Molecular analysis was performed on a representative tip from each unique morphotype within the depth layer of a core in a given year. While multiple sampling from a morphotype pile is generally preferable, the core depth partitioning limited the sampling space effectively enough that morphotype piles were generally small (1.6 mg ± 5.0 SD, or *c.* 4–10 root tips) which reduced the likelihood of missing species. The validity of this assumption was supported by obtaining identical DNA sequences from multiple tips within 10 different morphotype piles. Freeze-dried tips were crushed with a 35 mm glass bead (Fisher Scientific, Santa Clara, CA, USA) in a mini bead-beater (Biospec Products, Inc., Bartlesville, OK, USA) for 25 s and suspended in 1000 µl CTAB/PVPP buffer (2% CTAB, 1% PVPP, 0.1 M Tris pH 8.0, 1.4 M NaCl, 0.02 M EDTA). Following a 60-min incubation at 65°C, samples were vortexed with 600 µl chloroform:isoamyl alcohol (24 : 1) and centrifuged (13 000g) for 5 min. Samples were further cleaned with Qiagen DNeasy genomic isolation kits and resuspended in 50 µl AE buffer (Qiagen Inc., Valencia, CA, USA). PCR was performed in a PTC-100 thermal cycler (MJ Research Inc., Waltham, MA, USA) in conditions previously described (Gardes & Bruns, 1993) with 5 µl of the isolated nucleic acids in a 50 µl reaction. The internal transcribed spacer (ITS) region was amplified with the fungal-specific ITS1F (Gardes & Bruns, 1993) and ITS4 (White *et al.*, 1990) primer pair that targets both ascomycetes and basidiomycetes. PCR reactions were carried out with the addition of fluorescently labeled dNTPs (PE Biosystems, Foster City, CA, USA) to internally label the PCR product. Aliquots of each sample were digested separately with the restriction enzymes *Hin*I and *Alu*I (New England Biolabs, Inc., Beverly, MA, USA) for 3 h. Excess nucleotides from each fluorescent RFLP (fRFLP) were removed by precipitating with 0.5× vol 3.0 M NaOAc pH 4.6 and 2.5× vol 95%

EtOH, and rinsing with 70% EtOH. Raw data on fragment separation was collected on an ABI377 DNA fragment analyzer and analyzed using Genescan 3.1 software (PE Biosystems, Foster City, CA, USA). Whereas agarose gel resolution tends to have a roughly 10% error in size calling (Kåren *et al.*, 1997), we estimated gel-to-gel error rates to be roughly 1% of the size of the fragment with our internal labeling approach. We therefore used 1% as the cut-off for calling identical bands. Each unique fRFLP type within a plot in a given year was sequenced using a single pass of the ITS1/5.8S/ITS2 region. As samples with multiple fungi can produce false RFLP patterns that would inflate diversity estimates, this approach allowed validation of each type as well. To confirm the ability of fRFLP to distinguish species-level distinctions, multiple samples of each fRFLP type were sequenced. While the fRFLP analysis was used to initially screen the samples, the final species groups were based entirely on DNA sequence similarity. DNA sequences were compared both internally and externally to distinguish species-level 'sequence types'. Taxonomic affinities of the fRFLP types were initially identified by using BLAST searches (Altschul *et al.*, 1997) with the total obtained sequence to allow as accurate a taxonomic label as possible on the sample. A more detailed alignment was then carried out on subsets of sequences that had similar taxonomic affinities.

Samples from 1999 and 2001 were initially analyzed using the fRFLP approach described above, however, before the 2002 sampling, sale of the fluorescently labeled nucleotides was discontinued. A DNA sequence-only approach was therefore taken for the final year and reapplied to the 1999 and 2001 years as well. The primer set ITS1F and ITS4 was used to amplify PCR product from the original DNA isolation for sequencing. Because DNA sequencing is sensitive to the presence of multiple fungi, such as ascomycetous endophytes, any samples that did not sequence well due to multiple fungi were reamplified from a 10-fold dilution of the original DNA isolation. If there was still background signal then the primer set ITS1F and ITS4B (Gardes & Bruns, 1993) was utilized. To test the validity of this approach we sequenced any PCR product obtained from both primer sets used on an additional 24 root tips. There were no instances of obtaining different sequences from the same root tip indicating that there was no systematic bias against detecting basidiomycete taxa. Probably due to degradation, samples from 1999 did not reamplify well. As the fRFLP data from 2001 did not conflict with the DNA sequence data, the final dataset for the 1999 sampling included results from both screens.

Due to the large quantity of sequences, only the ITS1 region was used in the full alignment comparison. From this alignment we used PAUP v4.0b10 (Swofford, 2002) to calculate pairwise sequence distances with gaps and missing data excluded. Two sequences were considered to be from the same sequence-type if they were < 4% different. This percentage was chosen based on single-pass sequence error rates empirically

estimated to be between 0.5 and 1.0%, estimated distances of observed sister taxa in other community studies (A. Izzo, unpublished), and reports of up to 1.5% sequence difference within *Tricholoma flavovirens* across a 7 km distance (Horton, 2002). An exception was made in the case of sequences matching the genus *Cortinarius*. This group has proven difficult to resolve by RFLP analysis (Kåren *et al.*, 1997) and therefore we assumed that a lower distance (3%) would be a better representation of species-level divergence.

Taxonomic names were assigned to species types based on first the reliability of the name on the GenBank sequence, and second the strength of the BLAST match. To minimize background noise due to conserved regions, only the highly variable ITS1 spacer region was used in the BLAST analysis. Whenever possible, we considered a strong match to a database sequence that originated from a sporocarp, and that had been identified to genus, as the top match. To get an objective measure of the strength of the match we developed a simple weighted index based on first what portion of the query sequence showed a detectable match, and second the percentage of base pairs that matched across that portion. A weighted average (range 0–100) of these two values was calculated $((2x + y)/3$ where x is the lower of the two scores and y the higher). To establish appropriate cutoffs for taxonomic designations, we performed a test analysis using the Suilloid lineage. This group includes the sister genera *Suillus* and *Rhizopogon*, which are two relatively distinct morphological genera and are among the best-represented ectomycorrhizal lineages in GenBank. BLAST analysis of the ITS1 sequence of two different *Rhizopogon* spp. indicated that the lowest-scoring matches to *Rhizopogon* spp. that were still better than matches to *Suillus* spp. were roughly 90% similar across 67% of the query sequence, giving an index score of 75. Any sequence that matched a named GenBank sequence at that level or higher thereby was considered to be within that genus. However, if there were multiple genera that were equally strong matches, or the distinctions between genera in those lineages are known to be problematic, then these sequences received family or higher taxonomic labels. The final name given in the study therefore indicates a sequence that is at least 3–4% different from other sequences in the study.

DNA analysis of ECM host plant for taxonomic identification

We chose to use DNA techniques to confirm the host root composition because the core size of the soil samples was relatively small and often left very little root as reference to identify the host. To identify the host species, the tRNA spacer region of the host chloroplast genome was amplified using the primers trnTa and trnLb (Taberlet *et al.*, 1991) on a subset of ECM tips collected in 1999 across *Abies* spp. stands both from this study and a preliminary description of the ECM (North *et al.*, 2002). RFLP band patterns were obtained by a single restriction digest of the PCR product using *Hin*fI performed according to manufacturer's suggested conditions (New England Biolabs, Beverly, MA, USA). Bands were visualized by electrophoresis on a 3% agarose gel that was stained with ethidium bromide and viewed with UV light. GelReader software (NCBI) was used to size the fragments. The resulting patterns were unique to our primary ECM host species – *Abies concolor* (480 bp uncut; 340 and 80 cut), *Abies magnifica* (480 bp uncut; 180, 160, and 80 cut), *Pinus jeffreyi* (500 bp uncut; 210, 130, 80 cut), and *Pinus lambertiana* (470 uncut; 180, 110, and 80 cut). RFLP band size totals do not match up to the uncut band size due to the difficulty in viewing bands lower than 100 bp.

Data analyses

To measure the plot-level stability of ECM biomass we calculated SEM: mean ratios for the biomass at each depth in each plot, and performed a one-way ANOVA following testing for equal variance (Bartlett's) and normality (Shapiro-Wilk).

Two similarity indices were calculated to highlight different aspects of community similarity. The Sorenson index of similarity (Magurran, 1988) compares species presence/absence and stresses shared taxa between sites, whereas the Steinhaus index can be used on raw abundance data and is sensitive to large changes in the dominants (Legendre & Legendre, 1998). The formula for the Sorenson index is $2a/(2a + b + c)$ where a = the number of species shared, b = the number of species unique to plot 1 and c = the number of species unique to plot 2. The formula for the Steinhaus index is $2x$ (sum minimum abundances for each species)/(sum of abundances for both sites).

For the tests using the Sorenson index, rarefaction was used to adjust ECM richness to equal biomass levels within cores for each plot, between plots, and by depth. For example, if rarefaction estimates indicated there should be three fewer species in a core, we removed three species randomly. The chance of a species being chosen for removal was weighted to account for its relative biomass. The final biomasses used were adjusted as follows: 2.5 mg for an entire core, 1.0 mg when analyzing the depths within cores, 15.2 mg for an entire plot, and 3.7 mg for depths within plots. The biomass totals were chosen to maximize the number of cores and the number of species included and ultimately represented 70–80% of the species in the unadjusted dataset. Cores with zero or very low biomass were excluded from the final analyses. We estimate that there are roughly 2–6 root tips for every 1.0 mg dry root biomass.

We used Partial Mantel tests to isolate the overall effects of space and time on the similarity between cores. To determine more specific structure, we used autocorrelation tests with distance classes that would isolate within-plot comparisons (i.e. < 25 cm) from between-plot comparisons (over 20 m). Both the Partial Mantel and the autocorrelation tests were

performed using the program R 4.0 (Casgrain & Legendre, 2001). The differences we measured in ECM biomass across depths and years suggested that the ECM community structure would also be differentially affected. Therefore, we repeated these analyses first after stratifying the ECM community of the cores into an upper portion of the soil (any organic layer combined with the upper 10 cm of mineral soil) and a lower portion (the lowest 10 cm of mineral soil), and second within each of the sampling years (entire cores only).

To test for any fine-scale effects of time on core-core similarity within a plot, we compared cores located only 5 cm apart within and between years using a nonparametric test (Wilcoxon ranks). For this analysis, core composition was adjusted based on rarefaction to a common biomass of cores within the same plot.

To compare community composition within, and among, plots over time, we performed detrended correspondence analysis (DCA) on the total plot ECM communities across similar time points (2001 and 2002). Total ECM communities were included for each plot, and each species was adjusted to a relative abundance. The analysis was performed in PC-ORD v4.0 (McCune & Medford, 1999) with rare species downweighted based on their relative abundances.

We compared differences in species richness across depth using paired t-tests of the richness in each depth in a plot following rarefaction of species richness to that expected in 3.7 mg dry root biomass.

Results

Biomass of total active ECM roots

The amount of ECM biomass at the plot level varied considerably across the compartment, however, biomass at the lowest depth appeared to be more consistent across space and time. The average SEM : mean ratio at a given plot across years was significantly less in the lower mineral soil (ANOVA $P = 0.0002$) indicating that ECM biomass was more stable between years at this depth. ECM biomass was significantly lower in 1999 in the upper portion of the soil (sum of organic and the top 10 cm of the mineral horizon) than in other years while no change was detected in the lower portions (ANOVA, $P < 0.0001$, Tukey-Kramer HSD test) (Fig. 1). Only three plots (UN14, UN18, and UN27) had substantial ECM biomass in the organic layer (maximum of 12.7, 31.9, and 4.4 mg in a year, respectively), and in all but one of these plots there was no ECM biomass in 1999.

Molecular identification of ECM fungus and host from root tips

Of the 1300 ECM root tips analyzed, we were successful in PCR-amplification and BLAST identification of approx. 65% of the samples representing at least 80% of the biomass from each year. More than 150 fRFLP types were originally identified, but sequence analysis allowed us to identify many as resulting from mixed colonizations or only differing from each other by 1–2 bp. Mixed samples were removed from the final dataset, and the unique fRFLP types that were < 4% different were considered to be one type. Following the DNA sequence analysis we identified a total of 101 taxa (see Table S1, available online as supplementary material). Due to limitations of the database for ITS sequences, a number of taxa could not be resolved beyond phylum or class. In these cases, while the best BLAST match is reported, there was no clear lineage shared by the collective of best matches.

Of roughly 300 ECM root samples PCR-amplified for identification of the host, 227 successfully amplified on the
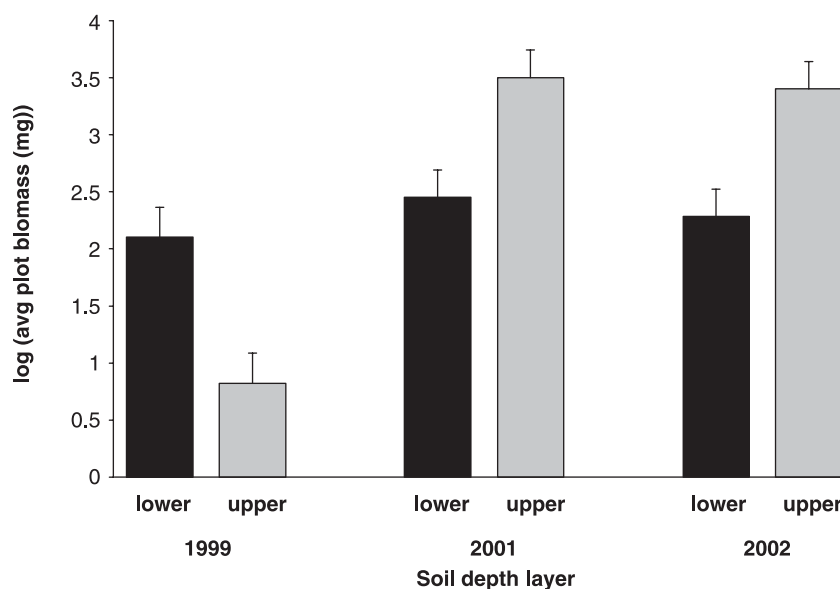

Fig. 1 Dry weight ectomycorrhizal (ECM) fungal biomass in plots across years and depths within mineral soil. Biomass is summed across all cores within the year in the lower 10 cm of mineral soil ('lower') or the sum of the organic layer and the upper 10 cm of mineral soil ('upper'). Different letters indicate significantly different biomass ($P < 0.05$ Tukey-Kramer HSD).

Portion of core included	Variable	Similarity measure	
		Sorenson	Steinhaus
Upper mineral + organic layer	distance	$r = -0.117^{**}$	$r = -0.096^{**}$
Lower mineral layer	distance	ns	$r = -0.085^*$
Entire core	distance	$r = -0.179^{***}$	$r = -0.145^{***}$
Upper mineral + organic layer	time	$r = -0.066^*$	$r = -0.043^*$
Lower mineral layer	time	ns	ns
Entire core	time	ns	ns

All tests compare only the samples collected in early summer (2001 and 2002) and compare cores at all scales across the nine plots. Before analysis using the Sorenson index, the composition of each core was adjusted by rarefaction based on similar biomass across all plots (2.5 mg for entire core, 1.0 mg for depths within core). Analyses using the Steinhaus similarity index were performed on raw abundance data. Negative Mantel r coefficients are indicative of a reduction in core-core similarity across space. The significance of the Mantel r coefficient is based on 9999 permutations.
*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not signifcant.

first attempt and were used in the RFLP analysis. While the majority of roots were from the two *Abies* spp. (89.4% *A. concolor*, and 4.4% *A. magnifica*), *P. jeffreyi* (2.2%) and *P. lambertiana* (4.0%) were also detected. Because of the low frequency of pine roots and the dominance of fungi capable of colonizing multiple hosts in other California forest systems (Horton & Bruns, 1998; Cullings *et al.*, 2000; Kennedy *et al.*, 2003), no impact was expected on the final results.

Spatiotemporal structure

A large number of taxa were only detected locally and during one sampling timepoint (see Table S2, available online as supplementary material). Almost three quarters (71%) of the taxa were limited to a single plot. Of these, *c.* 70% (51 total) were only detected in a single year, and *c.* 60% (41 total) were restricted to a single core in a single year. Roughly a quarter (23%) of the taxa were detected in the same plot across years. In seven of the 28 instances where a species occupied three or more cores in a plot during 1 yr, that species was not found in that plot at all the following year (see Table S2, available online as supplementary material). This pattern occurred across six of the nine plots, and twice in the case of one species (Russula4). Of the 30 instances where a species occurred in two cores in a plot during a year, 16 were not detected the following year. Tricholoma1 accounted for two of these instances and various Thelephoroid species accounted for five. The only species that occupied multiple cores at a plot between years included *Cenococcum geophilum* (eight of the nine plots), Wilcoxina1 (3 times), Piloderma2 (three times), Agaricoid3, Basidiomycota9, Thelephoroid5, and Thelephoroid13. The five most frequent taxa in cores and plots were *Cenococcum geophilum* (65 cores/nine plots), Wilcoxina1 (30 cores/eight plots), Basidiomycota9 (12 cores/six plots), Basidiomycota8

(18 cores), Tricholoma1 (12 cores/six plots), Ascomycota1 (plots plots).

We were able to detect low levels of both spatial and temporal structure in the ECM community analyzed by Partial Mantel tests (Table 1). This overall structure is driven by the autocorrelation of cores within a plot (Fig. 2). Similar to the pattern seen with the overall structure, the autocorrelation patterns also were stronger in the upper portion of the soil. The strength of the autocorrelation, however, was not equal across years, and was reduced in the late-summer sampling (1999). Temporal change of community similarity at the small scale (cores within 5 cm of each other) was detected as well with a significant reduction across years (Wilcoxonan rank sum test $P = 0.044$).

While there were exceptions, plots did not change dramatically relative to other plots from year to year as indicated by DCA (Fig. 3). The overall community across our plots does not appear to be changing dramatically based on the presence or absence of the dominant species either: most of the dominant ECM fungi across all plots in a given year were generally detected in other years (Fig. 4). The overall composition at a plot was also comparable between years. The similarity of the ECM community within a plot between years (0.45 ± 0.10 SEM, range 0.31–0.64) was significantly higher than between plots (0.18 ± 0.01 SEM, range 0–0.45)(ANOVA, $F = 53.6$, d.f. $= 90$, $P < 0.001$), although this varied by plot (Fig. 3).

More species were found on average in the upper portion of the soil (samples adjusted by rarefaction, 6.77 spp. per plot ± 0.46 SEM, upper 10 cm mineral soil and organic layer combined) than in the lower 10 cm of mineral soil (5.35 spp. per plot ± 1.73 SEM). The difference in richness was significant when plot-level differences were accounted for (samples adjusted by rarefaction, paired t-tests, $P = 0.03$).
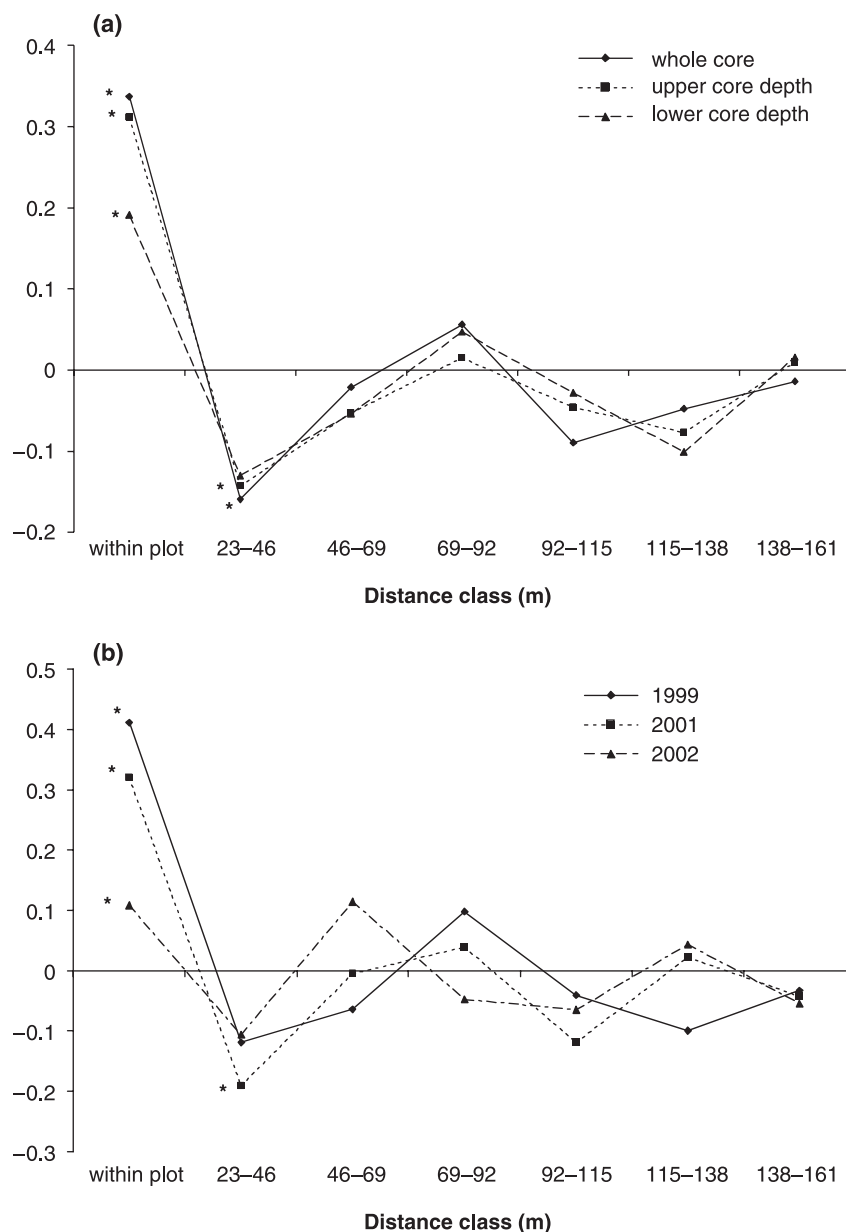
(a)

(b)

Fig. 2 Mantel autocorellograms of core-core composition based on the Sorenson similarity index. Autocorellograms are shown with comparisons across (a) depth of soil layer, or (b) year sampled. Similarity index was calculated following rarefaction of each core to equivalent biomass (2.5 mg dry biomass or *c.* 5–15 root tips per core for 2001 and 2002, 1.0 mg for depth comparisons, and 0.5 mg for 1999). Only samples from comparable time point samplings (2001 and 2002) are included in the depth analysis. Distance classes that have significant similarity (positive values for Mantel's *r*) or dissimilarity (negative values) following Bonferoni adjustment are indicated by an asterisk ($P < 0.05$).

Discussion

Spatiotemporal patterns

The ECM root community in our study was not static at small scales across years. Evidence for this statement includes changes in within-plot autocorrelation across years (Table 1) as well as a decrease in core-core similarity at small (5 cm) scales between years. Additionally, this temporal change was perhaps most clearly demonstrated by the frequent occurrence (six of the nine plots) of 'ephemeral' species that were located across three or four cores in a plot one year but were completely absent just 1 yr later only 5 cm adjacent. When we consider all species that occurred in at least two or

more plot cores in a given year, a large portion (40%) of them were not detected between years as well. Some of the apparent ephemeral behavior may be due to some physical disruption of the soil or roots due to sampling at such small scales, thereby leading to an overestimation of species turnover. At the same time, some of our species detected across years could also be different individuals, and thereby result in an underestimation of root community temporal change as well. Some studies of fungal genets demonstrate the possibility of overlap, and also show patterns suggestive of rapid spatiotemporal turnover such is seen in our study (Redecker *et al.*, 2001; Zhou *et al.*, 2001; Zhou & Hogetsu, 2002). The study of Redecker *et al.* (2001) is especially relevant to our study in that first it too was performed in a late-successional
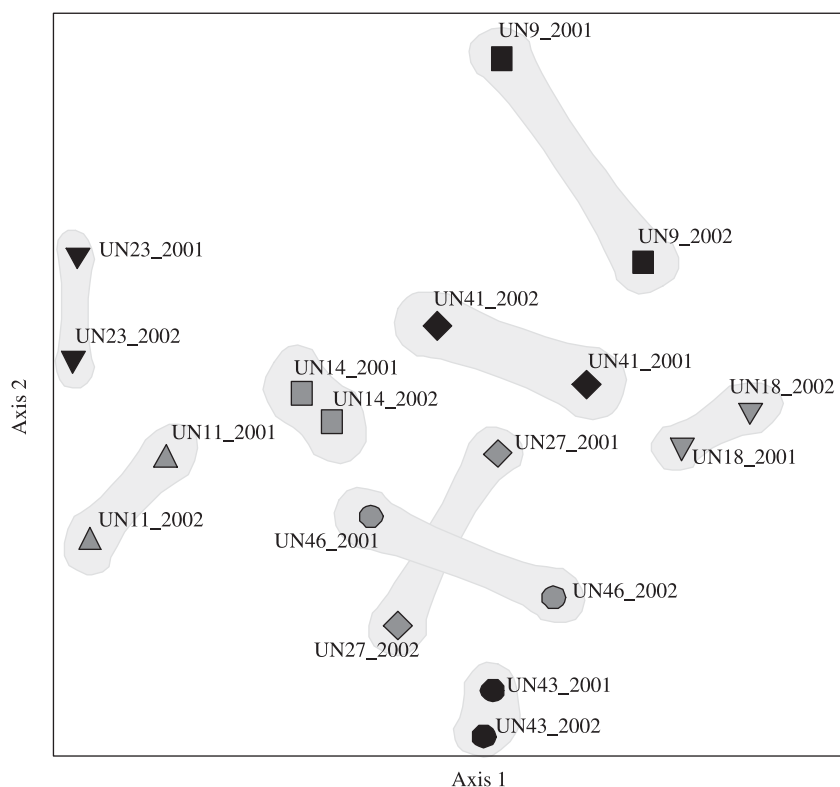
Fig. 3 Detrended correspondence analysis (DCA) of species occurrences in plots in 2001 and 2002. Species were adjusted to relative abundances within the plot before analysis. DCA was calculated in PC-ORD v4.0 with species impact weighted by its relative abundance. Axis 1 describes 9.8% of the variance and axis 2 describes 8.3%. Samples from the same plot in different years are connected.

forest with similar drought cycles, and second it focused on mature forest species (*Russula*, *Amanita*, and *Lactarius*) that are expected to inoculate roots primarily by previously established mycelium. In our study, the only fungus that exhibited clear annual turnover more than once also appears to be a Russula species (Russula4).

For ECM fungi to turnover locally across years, it would only require the turnover of roots coupled with a reduction in the likelihood of recolonization by the same fungi the following year. In our system there was a significant reduction in ECM root biomass in 1999 occurring primarily in the upper portion of soil (Fig. 1). Independent of whether this represents a seasonal turnover or strong annual variation, there are clearly periodic bottlenecks in the number of root tips available for ECM fungi to colonize, coupled with local mortality of some ECM species. At least some of this fine root turnover is due to their natural lifespan, which by recent estimates can range from one month to many years, and is probably quite variable (Hendrick & Pregitzer, 1992; Gill & Jackson, 2000; Gaudinski *et al.*, 2001). Life spans may also be affected by ECM status and potentially can vary in a species-specific manner (Treseder *et al.*, 2004). Environmental factors such as a reduction in water availability, however, can also accelerate root turnover (Swaty *et al.*, 1998). This may similarly affect the ECM in our system, as periodic drought is common in the Sierra Nevada and would be expected to dry the upper portion of the soil preferentially (Gomez *et al.*, 2002).

However, natural root turnover may also have a depth component (Hendrick & Pregitzer, 1996) independent of drying effects.

Regardless of how root turnover occurs, the disruption of the carbon supply that results is likely to cause additional dieback of the mycelium as is presumed in similar manipulative disturbance studies (Fleming, 1984; Simard *et al.*, 1997). The stress of soil drying in the summer may cause further mycelial dieback. Even if the mycelium community available for root inoculation does not change, the small-scale heterogeneity it exhibits, coupled with slight positional differences in where fine roots emerge, may be enough to add a stochastic element to what species colonize new roots. The possibility of fine root emergence acting as a small-scale disturbance in this way has been proposed as one mechanism towards maintaining diversity in ECM fungi (Bruns, 1995). Collectively these factors may explain why species richness was higher in the upper soil layers where root biomass was more variable across years.

While species composition fluctuated temporally at small scales, the ECM community was more stable at larger scales. The overall community within individual plots generally remained similar across years (Fig. 3), and most of the dominant species in any given year were detected in another year (Fig. 4). Thus the composition of dominant species in the ectomycorrhizal community appears to be stable across years, as would be expected in a late-successional forest. However, some species appear to be ephemeral locally, and many of the
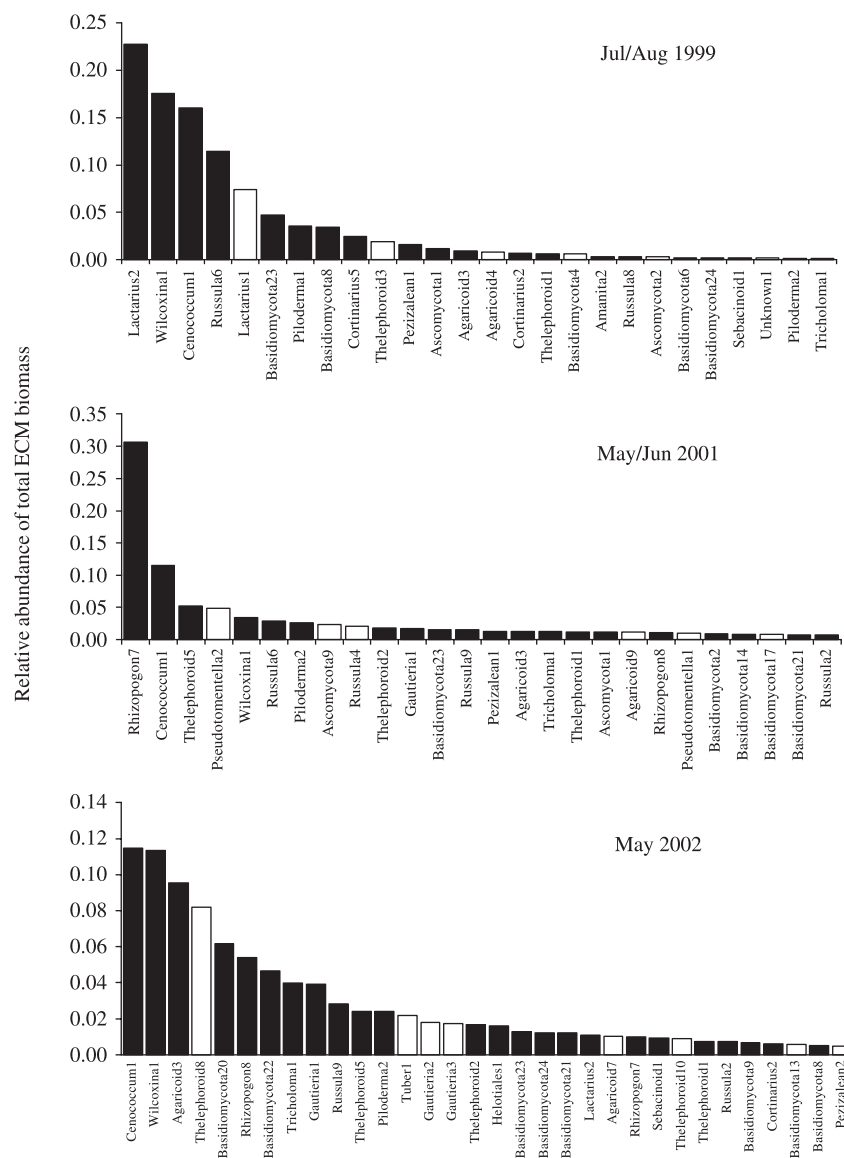
Fig. 4 Dominance diversity curves of ectomycorrhizal community for each sampled year. Relative abundance measures (the portion of dry root biomass that was identified by molecular means) for the most dominant species occurring across all plots within a sample year. Many species (46 in 2001 and 27 in 2002) with relative abundances below 0.02 are not shown. Black bars indicate species that were detected across multiple years. Note that the Y-axis scale differs between years.

dominant species vary dramatically in relative abundance among years. These patterns appear to be similar to the case with *Suillus grevillei* in which individual mycelia grow through a space in a season, and therefore vary locally across time, while remaining common at a broader spatial scale. (Zhou *et al.*, 2001).

The observed spatial structure in the ECM root community was driven by the higher similarity of within-plot core comparisons and appears to be temporally variable. The cohesion of the ECM community within small plots is reminiscent of that seen in other studies in which similarity of ECM root communities is above background level only within distances < 4 m (Jonsson *et al.*, 2000; Tedersoo *et al.*, 2003; Lilleskov *et al.*, 2004) and where the dominant species vary between trees (Gehring *et al.*, 1998). Autocorrelation at this scale, however, is reduced in 1999 (Fig. 2) where the sampling was

performed further into the summer drought. We think that this pattern is caused by the reduced biomass of roots in the upper portion of soil in 1999. This reduction in biomass limited the community to that in the lower portion of the soil that appears to be less structured overall (Table 1, Fig. 2). The reduction in locally dominant fungi, coupled with the persistence of *Cenococcum*, thereby made cores within plots as similar to each other as between plots.

Composition of the ectomycorrhizal root community

The taxonomic groups detected in our plots are generally very similar to those seen in other studies of the root communities (Horton & Bruns, 2001) with Russuloid, and Thelephoroid groups being the most speciose and collectively making up almost a quarter of the total species. Despite the fact that we

used molecular approaches that had the potential to identify taxa to species, many sequences could not confidently be matched to reliably named specimens in GenBank, a reflection of many underrepresented lineages still remaining in the databases (Horton & Bruns, 2001). The affinities of these other sequences were coarse but suggest that there are lineages somewhat related to the genera *Inocybe* and *Piloderma* that may account for another nine and 13 species, respectively. These affinities seem likely given that *Inocybe* is a very speciose genus in a forest dominated by *Abies alba* (Salerni *et al.*, 2000), and that many of the Athelioid taxa are prominent in root community studies (Horton & Bruns, 2001). *Cenococcum* was by far the most frequently detected type, a pattern that has been seen in many forests (Jonsson *et al.*, 1999; Peter *et al.*, 2001). The dominance and persistence of *Cenococcum geophilum* is also interesting to note because of the periodic drought this forest experience and because *Cenococcum* seems to increase in abundance in reduced moisture (Pigott, 1982).

Considerations for future studies

Some issues remain to be addressed in future studies. First, while our results suggest that seasonal drought could directly impact the ECM, our sampling cannot distinguish seasonal from annual variations. Studies examining ECM communities across contiguous seasons are therefore necessary to assess the real impact of the summer drought period. Second, while we have documented the turnover of species on active roots, it is possible that their hyphae remain active and are receiving carbon from another part of the genet that is still colonizing an active root elsewhere. The ability to consider multiple forms of the fungi will therefore greatly enhance our understanding of temporal change in ECM communities.

Acknowledgements

Supplementary material

The following material is available as supplementary material at http://www.blackwellpublishing.com/products/journals/suppmat/NPH/NPH1354/NPH1354sm.htm

Fig. S1 Schematic diagram of sample design.

Table S1 Results of BLAST analysis of fungal ITS1 sequences.

Table S2 Frequency of species occurrences across plots and years.

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